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REVIEW-SYMPOSIUM

Many kinases for controlling the water channel aquaporin-2

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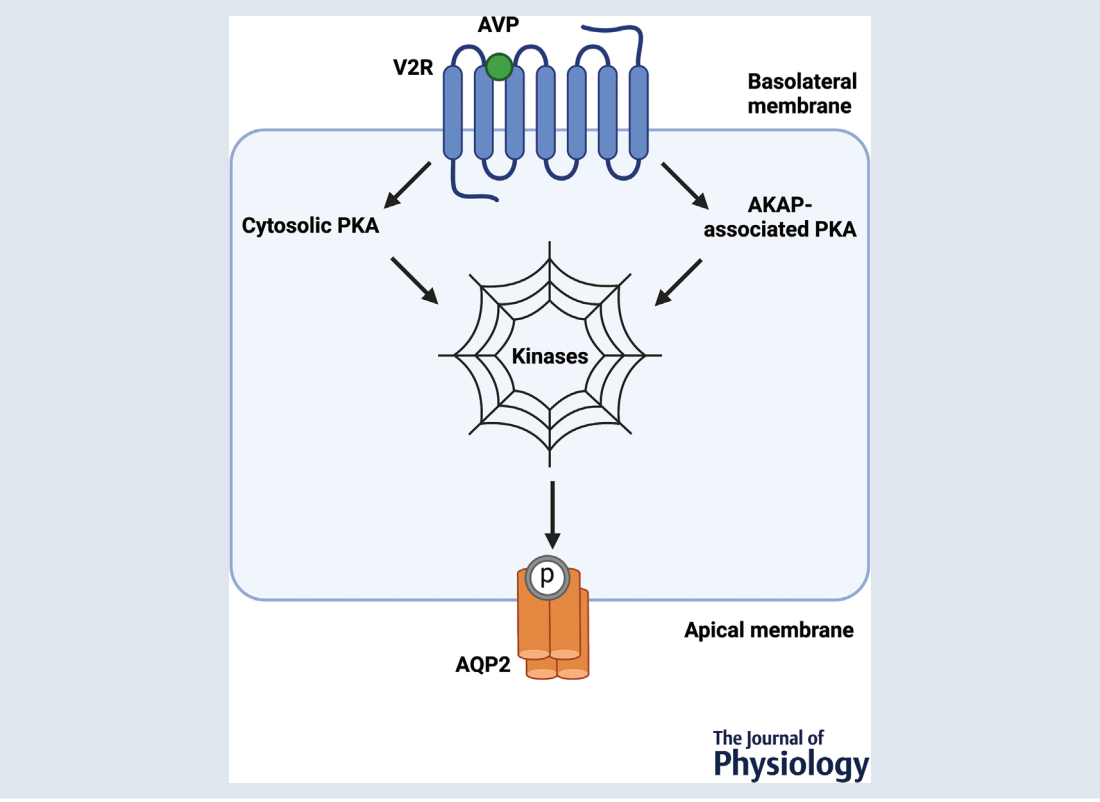

Andrii Kharin was born in Kyiv, Ukraine and moved to Germany for a Bachelor study in biochemistry at the Freie Universität Berlin. He carried out the practical part of his BSc in 2023 in the lab of Enno Klussmann where he characterised effects of novel small molecules on the localisation of AQP2.

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Abstract Aquaporin-2 (AQP2) is a member of the aquaporin water channel family. In the kidney, AQP2 is expressed in collecting duct principal cells where it facilitates water reabsorption in response to antidiuretic hormone (arginine vasopressin, AVP). AVP induces the redistribution of AQP2 from intracellular vesicles and its incorporation into the plasma membrane. The plasma membrane insertion of AQP2 represents the crucial step in AVP-mediated water reabsorption. Dysregulation of the system preventing the AQP2 plasma membrane insertion causes diabetes insipidus (DI), a disease characterised by an impaired urine concentrating ability and polydipsia. There is no satisfactory treatment of DI available. This review discusses kinases that control the localisation of AQP2 and points out potential kinase-directed targets for the treatment of DI.

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Abstract figure legend Arginine vasopressin (AVP)-mediated water reabsorption. AVP binding of vasopressin type 2 receptors (V2R) causes activation of a cytosolic pool of protein kinase A (PKA) and of an A-kinase anchoring protein (AKAP)-tethered pool of PKA. The activation of PKA, in turn, induces a network of kinases, which eventually leads to the accumulation of aquaporin-2 (AQP2) in the plasma membrane and thus water reabsorption from primary urine.

Introduction

Cells need to sense and integrate information from environmental cues such as neurotransmitters, hormones and chemical stimuli to adapt and react to changes of their environment through appropriate responses. The cellular responses to physiological cues are mostly triggered by stimulation of cue-specific receptors (Sholokh & Klussmann, 2021). The receptors often belong to the around 1000 seven-transmembrane G-protein-coupled receptors (GPCRs) or the around 100 receptor tyrosine kinases (RTKs). Activation of RTKs such as epidermal growth factor (EGF) receptors (EGFR) by EGF causes their dimerisation and autophosphorylation, which initiates the activation of downstream effectors; in the case of EGFR those include Ras/Raf proteins. Ras/Raf proteins, in turn, activate mitogen-activated protein kinases (MAPK), which phosphorylate downstream substrates. GPCRs couple to G proteins. Binding of an agonist to a GPCR causes the exchange of GDP for GTP on the G protein and thereby activates it. The binding of an agonist to a GPCR induces second messenger signalling. The second messenger depends on the G protein to which a GPCR couples. For example, GPCRs coupling to G_q signal through Ca^{2+} and GPCRs coupled to the stimulatory G protein (G_S) initiate cyclic adenosine monophosphate (cAMP) signalling. Ca^{2+} and cAMP, in turn, can both lead to activation of kinases; Ca^{2+} may activate calmodulin-dependent kinases (CaMKs) and protein kinase C (PKC), and cAMP activates protein kinase A (PKA). The kinases again phosphorylate and thereby modulate the activity of downstream effectors. An example of a process directed through a GPCR is arginine vasopressin (AVP)-mediated water reabsorption in renal collecting duct principal cells, which involves the tight control of the water channel aquaporin-2 (AQP2) through kinases and water channel phosphorylation. Thus, many different receptors and signalling cascades elicit cellular responses through kinases that function as molecular switches controlling on and off states of signalling cascade components and/or modulate their activity. Indeed, phosphorylation is one of the most common mechanisms of protein regulation.

This review will focus on the regulation of AQP2. It provides examples of insight into the complex kinase network and signalling that the control of a single protein requires.

Water reabsorption and aquaporin expression in the kidney. A major function of the kidney is the maintenance of body water homeostasis. The functional unit of the kidney is the nephron (Fig. 1). The kidney produces approximately 180 litres of primary urine daily, of which 90% is reabsorbed in the proximal tubule and Henle's loop. The remaining water is reabsorbed in the connecting tubule and collecting duct before approximately 1–2% of the originally produced primary urine is excreted. Water channels, aquaporins (AQP), mediate the water reabsorption (Fig. 1).

Humans express 13 AQPs. The orthodox AQPs (AQP0, 1, 2, 4, 5, 6 and 8) are water-selective, and the aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) allow for passage of water and larger uncharged solutes such as glycerol. Two AQPs, AQP11 and 12, are unorthodox AQPs that allow ions or gases to pass (Wu & Beitz, 2007). Early studies of the sequence of AQP1 by P. Agre and coworkers suggested a so-called hourglass model to reflect the membrane topology of an aquaporin

(Jung et al., 1994). The model has been confirmed for several AQPs by X-ray crystallography, e.g. for AQP2 at a resolution of 2.75 Å (Bai et al., 1996; Frick et al., 2014). AQPs are composed of six transmembrane domains (TM1–6) with intracellular N and C termini. TM1–3 and TM4–6 display obverse symmetry and fold in a way that two loops (loops B and E) come together to form a single aqueous pore through the lipid bilayer, basically corresponding to a seventh transmembrane domain (the 'hourglass'; Walz et al., 1997). Almost invariably each of the B and E loops contains an Asp-Pro-Ala motif (NPA box) at the centre of the pore (Xiong et al., 2023) (Fig. 2*A*). However, AQP7 possesses NAA and NPS motifs in the two loops. AQP11 contains an Asp-Ser-Cys (NPC) motif in the first box. The NPC box plays a role in oligomerisation of AQP11 (Ikeda et al., 2011). AQP12 contains NPT in the first box. The pore of orthodox AQPs measures around 2.4 Å in diameter, that of aquaglyceroporin >3.5 Å (Wu & Beitz, 2007). AQPs form tetramers, with each monomer providing a pore (Fig. 2*B*). Molecular dynamics simulations, fluorescence microscopic and stopped-flow light scattering approaches have revealed osmotic water permeabilities of AQPs: of AQP2 around 14×10^{-14} cm^3/s (Di Mise et al., 2019; Hadidi et al., 2021; Maric et al., 2001; Padhi & Priyakumar, 2017).

Of the 13 human AQPs, the kidney expresses eight: AQP1–4, 6, 7, 8 and 11 (He & Yang, 2019; Su et al., 2020) (Fig. 1). AQP1 is localised in the apical and basolateral plasma membranes of epithelial cells lining the proximal tubule and Henle's loop. It is responsible for most of the water reabsorption from primary urine, about 90%. AQP6 is expressed in the collecting duct (Su et al., 2020). It predominantly exists in intracellular vesicles, and is permeable for ions and water (Yasui, 2009). Water and ion permeability of AQP6 increased at acidic pH lower than 5.5, which is in line with its colocalisation with H^+-ATPase, a proton pump that acidifies secretory vesicles (Yasui, 2009; Yasui et al., 1999). AQP7 is expressed in the proximal tubule and AQP8 is found in both proximal tubule and the collecting duct (Su et al., 2020). AQP11 is localised intracellularly, mainly in the endoplasmic reticulum, and involved in the control of renal oxygen homeostasis (Ikeda et al., 2011; Qiu et al., 2023).

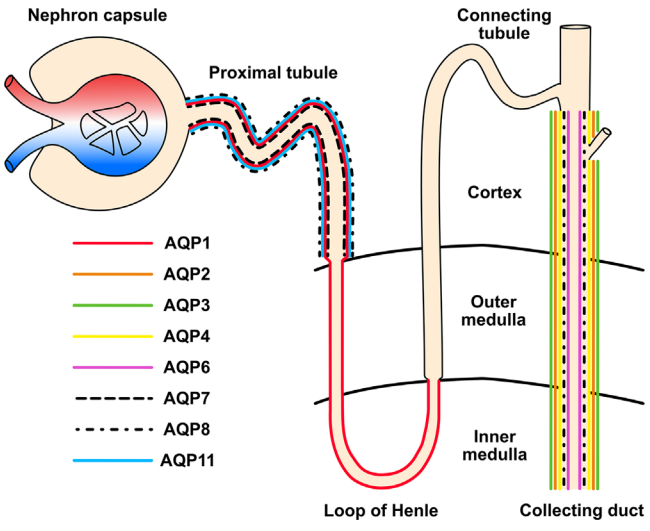

Figure 1. Schematic representation of a nephron, the functional unit of the kidney
Indicated are nephron segments and expression of aquaporins (AQPs).

AQP2, AQP3 and AQP4 are expressed in collecting duct principal cells. Through the principal cells, around 10% of the water from primary urine is reabsorbed. This water reabsorption is controlled by the peptide hormone AVP (Centrone et al., 2022; Klussmann, 2023; Vukicevic et al., 2016).

AVP-mediated water reabsorption in collecting duct principal cells. AVP is produced in the hypothalamus and released into the blood stream in the posterior pituitary gland in response to increased blood osmolality. AVP then stimulates its cognate vasopressin type 2 receptors (V2R) on the basolateral surface of collecting duct principal cells (Fig. 3*A* and *B*). The V2R is a GPCR which couples to the stimulatory G protein, G_s. AVP stimulation of V2R activates G_s, which, in turn, activates adenylyl cyclases (AC) to synthesise cAMP. AC6 is the main adenylyl cyclase in the collecting duct (Rieg et al., 2010). The AVP-induced increase of cAMP causes the cAMP to bind to the regulatory subunits of protein kinase A (PKA), promoting the dissociation of catalytic subunits from the regulatory subunits. The dissociation activates the C subunits, which phosphorylate their substrates (Ramms et al., 2021; Walker-Grey et al., 2017). However, there is also evidence that PKA holoenzyme can be active (Smith et al., 2017). Activated PKA initiates signalling that causes the redistribution of AQP2 from intracellular vesicles into the plasma membrane. The plasma membrane insertion of AQP2 facilitates water reabsorption by the principal cells. Along an osmotic gradient, water enters the cells through AQP2 and exits the cells through AQP3 and AQP4, which are constitutively located in the basolateral plasma membrane (Centrone et al., 2022).

Phosphorylation of AQP2. The induction of cAMP/PKA signalling upon binding of AVP to the V2R indicates the involvement of protein phosphorylation in the regulation of AQP2. Proteomics and phosphoproteomics studies mainly driven by Knepper and coworkers uncovered effects of AVP on the phosphorylation status of AQP2 and revealed networks of involved kinases and kinase substrates (Salhadar et al., 2021; Yang et al., 2022). Such analyses identified four phosphorylation sites (Fig. 2*B*),

serine (S) 256, S261, S264 and S269, in the C terminus of AQP2 (Hoffert et al., 2006) whose phosphorylation status changes upon AVP stimulation and which play key roles in the regulation of the AQP2 localisation and thus water reabsorption.

S256 lies inside of a PKA consensus sequence, RRXS/TY, where X may be any amino acid and Y preferentially a hydrophobic residue. V2R/PKA activation leads to phosphorylation of AQP2 at S256 (Kuwahara et al., 1995), which initiates AQP2 plasma membrane accumulation. In several AQP2-S256D mutant cell lines, where S256D mimicked constitutive phosphorylation of this site, AQP2 accumulated in the plasma membrane even in the absence of AVP. Substitution of S256 for A256 or blocking V2R abolished the phosphorylation at this residue and the plasma membrane accumulation (Cheung et al., 2020). However, the role of V2R/PKA-induced phosphorylation of S256 is not black and white. In PKA-knockout mpkCCD mouse cells, the AQP2 expression was lost, underpinning the relevance of PKA in maintaining AQP2 expression. When AQP2 expression was reconstituted through transfection, it was still phosphorylated in the PKA-knockout cells, indicating an involvement of kinases other than PKA in AVP-induced AQP2 phosphorylation of S256. The relevant kinase/s has/have not been identified (Datta et al., 2020; Isobe et al., 2017). Protein kinase G (PKG) is a likely candidate. Its consensus site (R/K2-3-X-S/T) is similar to that of PKA, and PKG phosphorylated S256 of AQP2 in LLC-PK1 epithelial cells (Bouley et al., 2000) and primary cultured inner medullary collecting duct cells (Klokkers et al., 2009).

The phosphorylation of S256 precedes phosphorylation of other serine residues (Hoffert et al., 2006; Moeller et al., 2010). Phosphorylation of S264 and S269 is elevated upon AVP stimulation and occurs 3 and 4 min after cAMP elevation, respectively, whereas the S256 phosphorylation reaches its maximum already 41 s after the rise of cAMP. The S256A-AQP2 mutant was not phosphorylated at S264 and S269 (Hoffert et al., 2008). It was proposed that S269 phosphorylation contributed to plasma membrane retention of AQP2 (Hoffert et al., 2008), and S269 phosphorylation was associated with inhibition of AQP2 endocytosis (Wang et al., 2017). As one study did not find PKA to phosphorylate S269 (Hoffert et al., 2008) and two studies suggested that PKA phosphorylated this site (Hara et al., 2022; Isobe et al., 2017), it remains to be clarified whether PKA indeed can phosphorylate S269. The discrepancy may result from differences in experimental design. S269 phosphorylation plays a role in AQP2 trafficking independently of S256 phosphorylation (Cheung et al., 2019). For example, Src colocalises with AQP2 in mouse collecting duct cells (Lin et al., 2004) and Src inhibition caused S256-independent

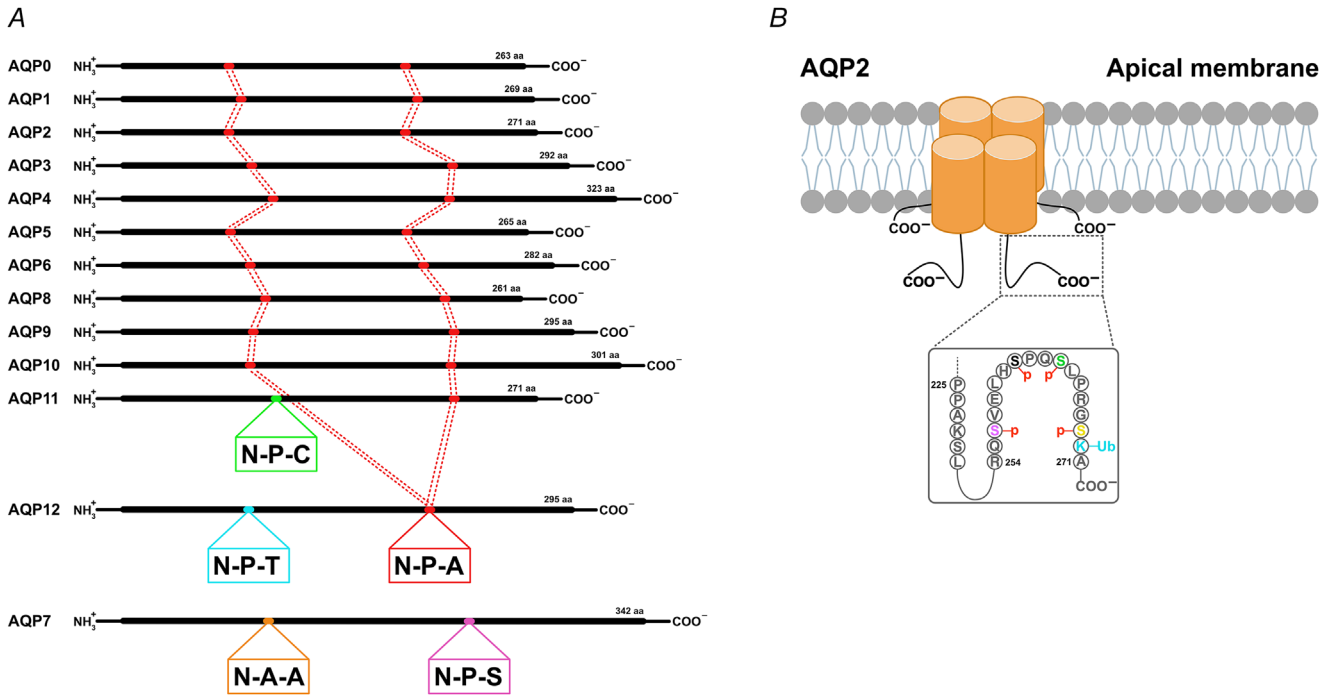

Figure 2. Conserved amino acids form the pore of AQPs
A, alignment of mammalian AQPs with motifs highlighted that are conserved within the cannel pores. aa, amino acid. *B*, AQPs form tetramers. The C terminus of rat AQP2 is highlighted. Indicated are the phosphorylation sites S256, S261, S264 and S269, whose phosphorylation status decides on the cellular localisation of the water channel. K270 is the target of channel ubiquitination.

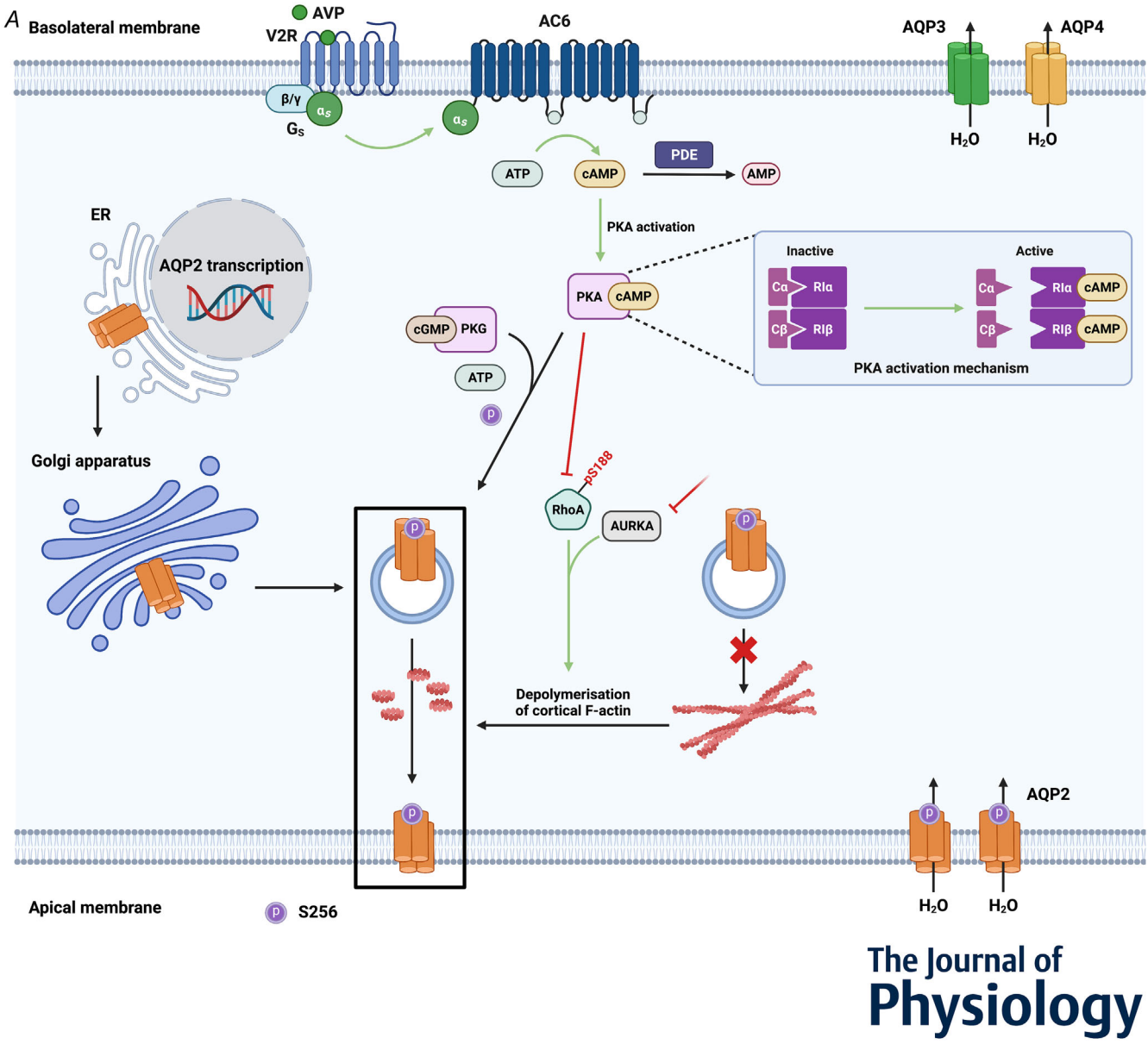

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Figure 3. AVP-mediated water reabsorption
A, antidiuretic hormone (arginine vasopressin, AVP) binding to vasopressin V2 receptors (V2R) on the surface of renal collecting duct principal cells stimulates the G_S-adenylyl cyclase (AC6) system, which increases the cAMP level. Two molecules of cAMP bind to each regulatory subunit (R) of protein kinase A (PKA), inducing a conformational change of the holoenzyme and thereby dissociation of catalytic subunits (C). The free C subunits phosphorylate their substrates, amongst them S256 of AQP2. The S256 phosphorylation increased the interaction of AQP2-bearing vesicles with F-actin. The F-actin cytoskeleton has a dual function. It provides tracks for AQP2-bearing vesicles to reach the plasma membrane, whereas peripheral F-actin acts as a barrier preventing AQP2-bearing vesicles to reach the plasma membrane. *B*, different pools of PKA control AQP2. A pool of PKA tethered to AQP2-bearing vesicles by the indicated A-kinase anchoring proteins (AKAPs) phosphorylates AQP2 at S256; whether the cytosolic PKA pool is involved in this phosphorylation is unclear. The cytosolic PKA pool phosphorylates CDK18 on AQP2-bearing vesicles. PKA activation induces downstream signalling by various other kinases. A few examples of kinases downstream of PKA activation are depicted in *A*; for details see text. Sites where AQP2 is phosphorylated are indicated by colour code. AVP stimulation of V2R eventually induces the accumulation of AQP2 in the plasma membrane and thereby facilitates water reabsorption along the osmotic gradient that is established in the kidney. The water enters principal cells through AQP2 and exits through AQP3 and 4 in the basolateral plasma membrane.

AQP2 plasma membrane translocation that was associated with S256-independent S269 phosphorylation (Cheung et al., 2019; Lu et al., 2004). Nephrogenic syndrome of inappropriate antidiuresis (NSIAD) can be caused by a R137L substitution in V2R, and is associated with constitutive plasma membrane localisation of AQP2. V2R-R137L induced Rho-associated protein kinase (ROCK)-mediated phosphorylation of S269 independently of S256 phosphorylation of AQP2 (Ranieri et al., 2020). Thus, even though phosphorylation at S256 is increased upon AVP stimulation, AQP2 does not necessarily have to be phosphorylated at S256 to be accumulated in the plasma membrane.

The role of S264 in the control of AQP2 is largely unclear. Phosphorylation of AQP2 at S264 is involved in recycling of AQP2-containing endosomes (Hoffert et al., 2008) and was found in human urinary exosomes (Sakai et al., 2020). The latter indicated plasma membrane localisation of AQP2 phosphorylated at S264, as exosome generation involves the plasma membrane.

Under resting conditions, AQP2 is phosphorylated at S261. This phosphorylation decreases after AVP challenge (Hoffert et al., 2006, 2007, 2008; Tamma et al., 2011). Phosphorylation of S256 is dominant over S261 phosphorylation as the S256D-S261D-AQP2 mutant, which mimicked constant phosphorylation at S256 and S261, was constitutively present at the plasma membrane even under basal conditions (Tamma et al., 2011). Phosphorylation of S261 likely occurs through MAPK kinases, extracellular signal-regulated kinase 1 (ERK1) and p38 MAPK, as their inhibition inhibited S261 phosphorylation (Centrone et al., 2017;

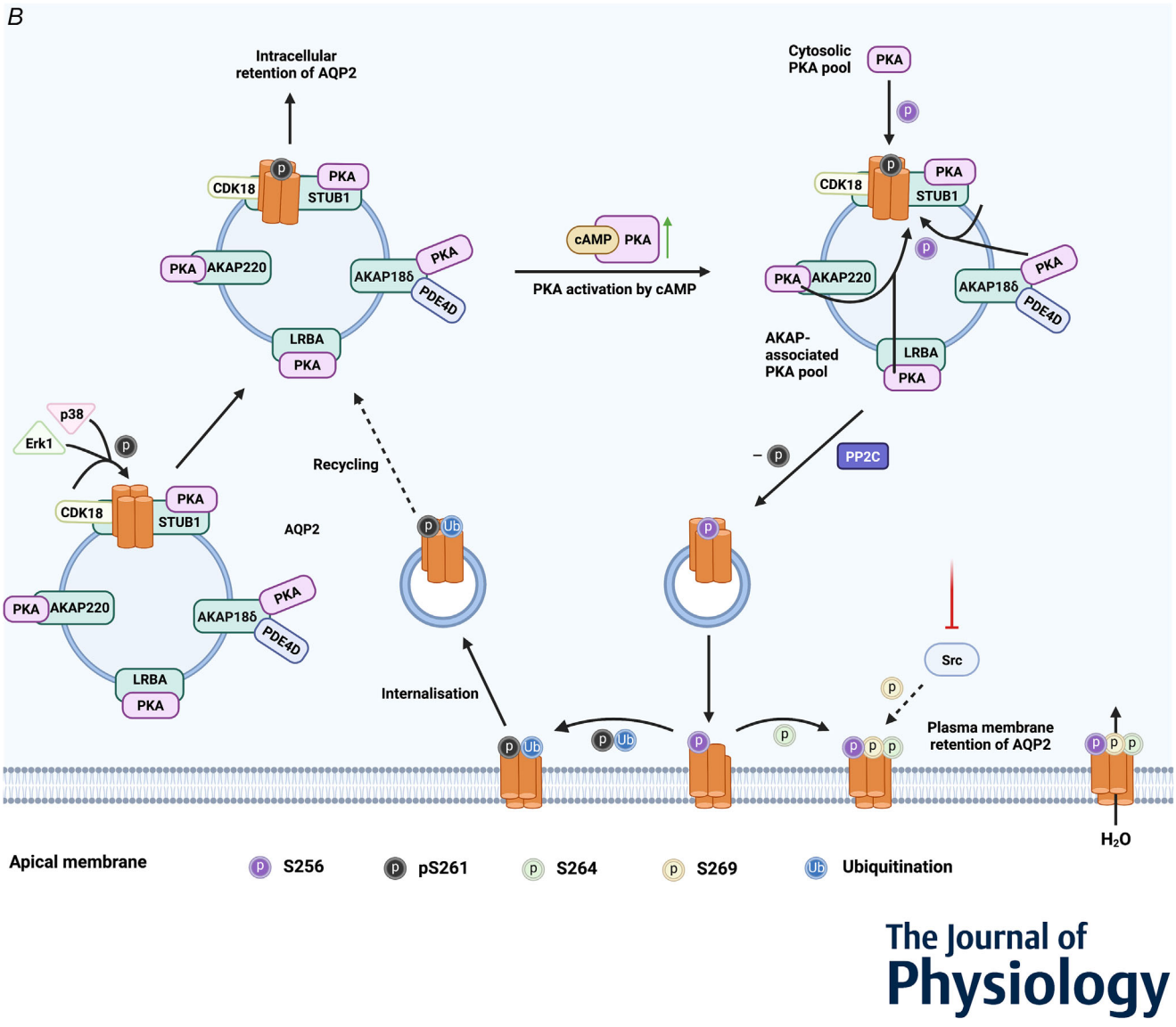

Figure 3. Continued

Cheung et al., 2017; Nedvetsky et al., 2010). Further kinases phosphorylating S261 are cyclin-dependent kinases (CDK) 1 and 5 (Tamma et al., 2014) and CDK18 (see below) (Dema et al., 2020).

Phosphorylated sites in proteins are dephosphorylated by protein phosphatases (PPs) to reset the proteins. Several PPs have been identified to dephosphorylate AQP2, including PP1, PP2A (Valenti et al., 2000) and PP2B (Deshpande et al., 2019). PP2C dephosphorylated pS261 in response to AVP stimulations (Cheung et al., 2017). In addition, PP2C decreased phosphorylation of ERK1, thus inhibiting it and contributing to the decrease in S261 phosphorylation (Cheung et al., 2017). The involvement of around 20 more S/T phosphatases has been suggested through proteomics and transcriptomics studies (LeMaire et al., 2017).

Kinase network downstream of V2R and links to F-actin. In addition to kinases directly catalysing the phosphorylation of AQP2, further kinases are involved in the control of the water channel. Several proteomics, phosphoproteomics and siRNA screening studies have revealed a whole network of kinases that are induced upon V2R stimulation, and a list of proteins they phosphorylate (Deshpande et al., 2019; Isobe et al., 2017; Leo et al., 2022; Park et al., 2023; Salhadar et al., 2020). The studies revealed that the phosphorylation of 33 sites on various proteins increased in response to AVP; 23 of those could be phosphorylated by PKA. AVP caused decreased phosphorylation of 18 sites on various proteins of which 15 contained proline; the decrease could be due to downregulation of MAPK activity (Yang et al., 2022). Moreover, analysis of V2R-induced, PKA-independent signalling using a proteomics approach indicated activation of the AMP-activated protein kinase (AMPK)/sucrose non-fermenting 1 (SNF1)-subfamily of the Ca^{2+}/calmodulin-dependent protein kinase (CaMK)-like family of kinases (Datta et al., 2020). However, an actual involvement of candidate kinases in the regulation of AQP2 identified by such approaches has not yet been verified.

Hypertonicity caused an accumulation of AQP2 in the plasma membrane of rat kidney collecting duct principal cells, an effect involving the activation of MAPK (p38 MAPK, ERK1/2, and c-Jun N-terminal kinase (JNK)) (Hasler et al., 2008). The activation of AMPK led to phosphorylation of AQP2. However, the phosphorylated amino acid residue and whether another kinase is involved are unclear (Klein et al., 2016; Klein et al., 2021).

A recent example of a kinase that controls AQP2 without directly phosphorylating it is Aurora kinase A (AURKA) (Baltzer et al., 2022). The knockdown of AURKA in a siRNA screen prevented the cAMP-induced AQP2 plasma membrane localisation in mouse collecting duct (MCD) 4 cells (Dema et al., 2020). The inhibitory effect was confirmed in MCD4 and primary rat inner medullary collecting duct (IMCD) cells using the AURKA-selective inhibitor Aurora-A inhibitor I and novel derivatives as well as a structurally different inhibitor, alisertib. Aurora-A inhibitor I and alisertib had opposite effects on F-actin stress fibres. While Aurora-A inhibitor I depolymerised F-actin stress fibres, alisertib caused their increase. The depolymerising effect of Aurora-A inhibitor I was consistent with the dephosphorylation of cofilin-1 (CFL1), which activates the actin-depolymerising function of CFL1. The alisertib-mediated increase of F-actin stress fibres was not associated with CFL1 phosphorylation. Thus, AURKA controlled AQP2 through different mechanisms.

The phosphorylation of AQP2 at S256 increased the interaction of AQP2-bearing vesicles with F-actin in cytosolic domains, and F-actin was considered to provide the tracks for AQP2 trafficking to the plasma membrane in response to AVP (Holst et al., 2021; Nedvetsky et al., 2007; Sasaki et al., 2014). Therefore, the inhibitory effect of Aurora-A inhibitor I on the AVP-induced redistribution of AQP2 may be explained by the removal of F-actin stress fibre tracks for trafficking. ROCK not only catalyses the AQP2 phosphorylation of S269 (see above) but also induces F-actin formation through phosphorylation of cytoskeletal elements. Inhibition of ROCK or its upstream activator RhoA caused an AVP-independent depolymerisation of peripheral F-actin and plasma membrane accumulation of AQP2 in primary IMCD and rabbit collecting duct cells. Thus, peripheral F-actin may serve as a physical barrier preventing AQP2-bearing vesicles from reaching the plasma membrane under resting conditions (Klussmann et al., 2001; Nedvetsky et al., 2007; Sasaki et al., 2014; Tamma et al., 2001). The barrier function would explain the alisertib-caused inhibition of the redistribution of AQP2 to the plasma membrane. However, not all AQP2-bearing vesicles reside below/in front of the peripheral F-actin layer; a sub-pool of AQP2-bearing vesicles resides between the F-actin layer and the plasma membrane and may constitute the pool readily available for fusion with the plasma membrane (Holst et al., 2021).

Location of kinases phosphorylating AQP2. The compartmentalisation of cAMP/PKA signalling is crucial for the regulation of AQP2 (Ando et al., 2023; Klussmann & Rosenthal, 2001; Klussmann et al., 1999; McSorley et al., 2006; Stefan et al., 2007; Vukicevic et al., 2016). PKA is tethered to cellular compartments by A-kinase anchoring proteins (AKAPs; Figure 3*B*) (Bucko & Scott, 2020; Dema et al., 2015; Sholokh & Klussmann, 2021; Vukicevic et al., 2016). Global disruption of AKAP–PKA interactions may

be achieved with high-affinity peptides, peptidomimetics or small molecules (Alto et al., 2003; Christian et al., 2011; Deak & Klussmann, 2016; Dema et al., 2015; Hundsrucker et al., 2006; Schäfer et al., 2013; Yu et al., 2014). Disruption of AKAP–PKA interactions with peptides prevented the cAMP-induced redistribution of AQP2 to the plasma membrane of primary IMCD cells (Klussmann et al., 1999; McSorley et al., 2006; Szaszak et al., 2008), and provided first evidence that not only PKA activity but also its location in principal cells matters.

Subsequent investigations identified AKAP18 (Henn et al., 2004; Henn et al., 2005; Stefan et al., 2007), AKAP220 (Okutsu et al., 2008; Whiting et al., 2016), STIP1 homology and U-Box containing protein 1 (STUB1; see below) (Dema et al., 2020) and lipopolysaccharide responsive and beige-like anchor protein (LRBA) (Hara et al., 2022) as anchors for PKA on AQP2-bearing vesicles in close proximity to AQP2. The AKAP-anchored PKA in close proximity to AQP2 phosphorylated S256 of AQP2 (Okutsu et al., 2008) and possibly S269 (Hara et al., 2022) (Figure 3*A* and *B*).

CDK18 had been identified in phosphoproteomics studies as a target of PKA upon AVP stimulation (Yang et al., 2022). Recently, a protein complex comprising CDK18, PKA, the ubiquitin ligase STUB1 (also known as CHIP), and AQP2 was identified in renal principal cells (Dema et al., 2020). The identification of the complex was initiated through a siRNA screen targeting 719 kinase-related genes that represented the majority of the kinases of the human genome. The screening identified 13 hits whose knockdown inhibited the cAMP-induced AQP2 plasma membrane localisation, as was shown by high-content imaging and biochemical approaches. Amongst the candidates was the so far hardly characterised CDK18. Further analysis revealed that STUB1 functioned as an AKAP tethering PKA to the CDK18/AQP2 complex on AQP2-bearing vesicles, where STUB1 bridges AQP2 and CDK18; both CDK18 and STUB1 controlled the plasma membrane insertion of AQP2; within the complex, they controlled AQP2 abundance, CDK18 through AQP2 phosphorylation at serine 261 and STUB1 through ubiquitination. AVP stimulation increased the PKA-catalysed phosphorylation of CDK18 and thus of its activity. The relevant pool of PKA phosphorylating CDK18 was cytosolic while the STUB1-anchored CDK18 phosphorylated AQP2 at S261 (Fig. 3*B*). Moreover, the STUB1-anchored PKA that is in a complex with AQP2 did not phosphorylate AQP2 at S256. Thus, two different pools of PKA are involved in the control of AQP2, a cytosolic and a compartmentalised AKAP-anchored pool, and only a subset of the AKAP-anchored PKA pool on AQP2-bearing vesicles facilitates PKA phosphorylation of S256 of AQP2. These data illustrate the delicate control of AQP2 by different pools of PKA.

Where in principal cells is AQP2 phosphorylated? Early studies had identified the endoplasmic reticulum and Golgi as sites of S256 phosphorylation (Procino et al., 2003). This may be the region where PKA phosphorylates AQP2, since in resting principal cells, a large fraction of AQP2-bearing vesicles resides in the perinuclear region and this is the region where PKA is activated first in response to AVP stimulation (Stefan et al., 2007). Recently, intracellular sites of S256 phosphorylation in LLC-PK1 cells were identified using inhibitors of the AQP2 recycling pathway (Cheung et al., 2023). The investigation revealed multiple sites along the recycling pathway, including the *trans*-Golgi network, and indicated that the protein complex required for S256 phosphorylation resided at various cellular locations involved in recycling.

Diabetes insipidus – potential treatment by targeting the kinase network? Dysregulated AVP-mediated water reabsorption causes water balance disorders, such as diabetes insipidus (DI), and is associated with various conditions where AVP-mediated water reabsorption is overactive, e.g. heart failure and liver cirrhosis (Olesen & Fenton, 2021; Vukicevic et al., 2016). AVP-mediated water reabsorption through principal cells involves three essential components: AVP, V2R and AQP2. The plasma membrane insertion of AQP2 reduces urine output and decreases plasma osmolality; inhibition of the plasma membrane insertion causes DI, characterised by polyuria and polydipsia (Christ-Crain et al., 2019).

Mutations in the genes encoding AVP, V2R and AQP2 cause congenital DI. In central DI (CDI), *AVP* gene mutations reduce or ablate serum AVP; the prevalence is 1 in 25,000 (www.orpha.net). V2R is encoded by the X-chromosome. Mutations in the *AVPR2* gene cause X-linked nephrogenic (N)DI, which accounts for approximately 90% of all congenital DI forms. The prevalence of X-linked NDI is 3.7 in 1 million males. The X-linked inheritance is more apparent in males due to the lack of a second X chromosome. In 10% of the cases, NDI is caused by mutations in the *AQP2* gene (Hinrichs et al., 2022).

The most common form of DI is acquired NDI. For example, lithium during treatment of bipolar disorders causes acquired NDI in up to 40% of the patients by inhibition of the AVP system in principal cells. Lithium inhibits glycogen synthase kinase 3 β (GSK3β) which, by modulating AC activity and decreasing cAMP, impaired AQP2 trafficking, reduced AQP2 mRNA and protein expression, and decreased its phosphorylation at S256 and altogether interfered with responses to AVP in the collecting duct principal cells (Kaiser & Edemir, 2020; Kishore & Ecelbarger, 2013; Rao et al., 2010). However, GSK3β knockout mice were only mildly resistant to lithium-induced DI – as were AC6 knockout

mice (Poulsen et al., 2017). Tolvaptan, a selective V2R antagonist, causes acquired DI during treatment of autosomal dominant polycystic kidney disease (ADPKD). ADPKD is caused by mutations in the genes *PKD1* and *PKD2* encoding polycystin-1 and 2 (PC1 and PC2). It affects 12.5 million people worldwide, and is characterised by a continued development and growth of cysts that invariably lead to end-stage renal disease (ESRD). Tolvaptan causes the loss of around 8 litres of hypotonic urine per day and polydipsia. These effects decrease the compliance for tolvaptan treatment and therefore promote progression of the disease (Olesen & Fenton, 2021; Torres & Harris, 2019; Zhou & Torres, 2022). If voiding could be reduced, compliance could increase due to decreased water loss, which would improve the outcome.

Apart from treatment of central DI by AVP substitution with an analogue, desmopressin, effective treatments of DI are not available. Thus, innovative approaches for the treatment are needed (Vukicevic et al., 2016). Any reduction of the water loss will not only reduce the frequency of voiding and thereby improve the quality of life of DI patients, but, most importantly, will ameliorate the side effects the water loss causes, which may not only be thirst but may be more severe, causing hypotension or seizure.

Targeting kinases of the network controlling AQP2 for modulating the localisation of AQP2 for therapeutic purposes would be attractive. Proteomics, phosphoproteomics and transcriptomics approaches have been used for analysis of animal models of diabetes insipidus (Mak et al., 2023). The detected changes in signalling pathways that involve kinases require validation of potential targets.

There are examples for interference with the kinase network controlling AQP2 to promote the plasma membrane localisation in cases of DI where AVP or V2R are dysfunctional but the principal cells possess the intact trafficking machinery. The approved antimycotic drug fluconazole is one. Fluconazole might have utility for treatment of diabetes insipidus (Vukicevic et al., 2019), and a clinical trial is ongoing (Eudra-CT clinical trial number: 2020-002204-38). Fluconazole promoted the plasma membrane insertion of AQP2 in mice in the presence of tolvaptan. It ameliorated the water loss and increased urine osmolality. Since it reduced the AQP2 phosphorylation of S261 and promoted the inhibitory phosphorylation of RhoA at S188, the drug may exert its action through modulating kinase activities (Vukicevic et al., 2019). Activation of AMPK with metformin did also induce the plasma membrane localisation of AQP2 in mice (Efe et al., 2016; Klein et al., 2016). Metformin and another compound, NDI-5033, which activates AMPK, improved the urine concentrating ability in rat and mouse models of acquired (tolvaptan- or lithium-induced) and congenital (V2R knockout) NDI. Thus, targeting AMPK seems a promising therapeutic approach for the treatment of diabetes insipidus (Klein et al., 2021; Tas & Sancak, 2021).

The utility of other agents that directly or indirectly target kinases for the treatment of NDI has been tested in rodent models and in a few patients. All at best have conferred short-term benefit: sildenafil (targets phosphodiesterase 5 (PDE5), indirectly activates PKG through increasing cGMP; Bouley et al., 2005; Sanches et al., 2012), erlotinib (EGFR antagonist and thus an RTK inhibitor; Cheung et al., 2016), statins (inhibit Rho and thereby ROCK activation; Li et al., 2011; Procino et al., 2014; Procino et al., 2016), ONO-AE1-329 (EP_4 prostanoid receptor agonist, can increase cAMP and activate PKA; Li et al., 2009) and clopidogrel (inhibits P_2Y_{12} and cAMP synthesis and thereby PKA; Zhang et al., 2015).

Due to the ubiquitous expression of kinases, targeting them would be prone to cause unwanted side effects. It might be a more promising strategy to modulate kinase location. Kinases such as PKA, PKC, PKD or CaMK and also phosphatases may be tethered to the relevant cellular compartments through protein–protein interactions, e.g. AKAPs; protein–protein interactions are excellent pharmacological targets due to their specificity and their possible cell type-specific occurrence (Bucko & Scott, 2020; Carlson et al., 2022; Klussmann & Rosenthal, 2008; Schächterle et al., 2015; Walker-Grey et al., 2022). So far, there are no examples for pharmacological interference with a specific protein–protein interaction that would support a utility for the treatment of diabetes insipidus. Only global uncoupling of PKA from AKAPs has been shown to prevent the plasma membrane accumulation of AQP2. This approach would be more suitable to target diseases where a predominant localisation of AQP2 in the plasma membrane promotes water reabsorption, such as in heart failure.

Conclusions

Although much detail of molecular mechanisms underlying the control of AQP2 has been uncovered, this has not yet led to new treatments of water balance disorder associated with its dysregulation. The kinase network, in particular renal principal cell-specific protein–protein interactions controlling the location of the involved kinases, presents potential targets for promoting the plasma membrane accumulation of AQP2 in diabetes insipidus. In response to AVP, AQP2 engages in other or additional protein–protein interactions as in resting cells. For example, in response to AVP, AQP2 engages in direct interactions with cytoskeletal proteins. Some interactions of AQP2 are controlled through its phosphorylation. Hsc70, hsp70-1, hsp70-2 and annexin

II bind less to S256-phosphorylated AQP2 than to AQP2 unphosphorylated at this site (Zwang et al., 2009). Such interactions may be explored as targets for the treatment of diabetes insipidus (Gao et al., 2020). Protein phosphatases potentially involved in controlling AQP2 through dephosphorylation have been identified (LeMaire et al., 2017). Their utility as targets for the treatment has not been evaluated.

Currently unanswered questions are why so many kinases control AQP2 and why there is redundancy in the kinases phosphorylating AQP2 at S256, a crucial initial step in its redistribution from intracellular vesicles to the plasma membrane. S256 is a site for phosphorylation by PKA, PKG and possibly a Golgi-resident casein kinase. S261 phosphorylation is also secured by several kinases, and similarly S269 (PKA and ROCK). One answer to why so many kinases control AQP2 may be that AVP-mediated water reabsorption is a vital process and water reabsorption and regulated urine concentrating ability are subject to modulation by multiple influences. The influences can be of completely different nature, ranging from hunger and stress to changes of blood pressure. All the influences are effective through stimulation of different plasma membrane receptors, which all initiate different downstream signalling cascades. Renal principal cells express amongst others prostaglandin, angiotensin II, EGF and Ca^{2+}-sensing receptors, exemplifying the range of stimuli the signalling systems of principal cells integrate. Downstream signalling in response to most, if not all, stimuli involves activation of kinases and crosstalk between kinases (Cheung et al., 2020). Such pathways may all contain potential targets, not just kinases, for the treatment of diabetes insipidus.

Future studies will need to reveal in detail where in the principal cells AQP2 is phosphorylated at which amino acid residue by which kinase and how the relevant kinases are tethered to their specific compartments. A detailed understanding of the kinase-dependent control of AQP2 will not only provide insight into the fundamental biological mechanisms controlling AVP-mediated water reabsorption, but may also provide novel targets for the treatment of diabetes insipidus and also diseases where AQP2 is predominantly in the plasma membrane, for example in states where AVP levels are elevated, e.g. in heart failure.

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Additional information

Competing interests

The authors have no competing interests/conflicts of interest in accordance with journal policy.

Author contributions

E.K. conceptualised and designed the manuscript. E.K. and A.K. drafted the work and revised it critically for important intellectual content. Both authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Supporting information

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